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PHAGOCYTE ACTIVATION AS AN INDICATOR OF SYSTEMIC INFLAMMATION IN THE NEWBORN INFANT

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Academic Dissertation

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Contents

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ABBREVIATIONS

a/A	alveolar-arterial
BPD	bronchopulmonary dysplasia
BPI	bactericidal permeability-increasing protein
CFU-GM	colony-forming unit granulocyte-macrophage
CI	confidence interval
CP	cerebral palsy
CRP	C-reactive protein
ELISA	enzyme linked immunosorbent assay
FIR	fetal imune response
FiO_2	fraction of inspired oxygen
FITC	fluorescein isothiocyanate
FMLP	formylmethionyl-leucyl-phenylalanine
GC	glucocorticoid
GCR	glucocorticoid receptor
G-CSF	granulocyte colony-stimulating factor
ICAM	intercellular adhesion molecule
IL	interleukin
IAI	intra-amniotic infection
IUI	intrauterine infection
IVH	intraventricular hemorrhage
LBP	lipopolysaccharide-binding protein
LPS	lipopolysaccharide
MIP	macrophage inflammatory protein
NADPH	nicotinamide adenine dinucleotide phosphate
NEC	necrotizing enterocolitis
NF	nuclear factor
NK	natural killer
PAF	platelet activating factor
PBS	phosphate buffer solution
PCT	procalcitonin

PE	phycoerythrin
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocyte
PROM	premature rupture of the membranes
pPROM	preterm premature rupture of the membranes
PVL	periventricular leukomalacia
RFU	relative fluorescence unit
sLEX	sialyl Lewis X
TNF-α	tumor necrosis factor α
TLR	Toll-like receptor

ABSTRACT

Background: Newborn infants are particularly susceptible to bacterial infections due to their immature immune functions. Diagnosis of bacterial infection is difficult because newborn infants present with unspecific clinical symptoms and because of the lack of reliable laboratory markers. In newborn infants, sepsis is the most common cause of systemic inflammation, which is characterized by activation of inflammatory cells, the coagulation system, and the complement system within the circulation. Signs of systemic and pulmonary inflammation are present also in preterm infants with respiratory distress syndrome (RDS). In the newborn infant, the immaturities of the host defence comprise qualitative and quantitative deficiencies of the innate and acquired immunity. Of the innate immune system, polymorphonuclear leukocytes (PMN), important cellular effectors that provide the major host defence against bacterial invasion, have been reported to function suboptimally.

Objectives: To study neutrophil CD11b and IL-8 as markers of systemic inflammation and sepsis in newborn infants with early onset sepsis, neutrophil CD11b expression as a marker of systemic inflammation in preterm infants with RDS, and the effects of dexamethasone on phagocyte activation in preterm infants with RDS. In addition, to study the ability of newborn PMNs to release granule contents.

Subjects: Newborn infants born at or near term with suspected sepsis (N=35). Healthy term infants (N=17). Preterm infants with (N=59) and without RDS (N=12). Cord blood samples from preterm (N=21) and term infants (N=30) were obtained for *in vitro*-stimulation assays.

The entire project comprised 174 newborn infants. From 51 of these infants, only cord blood was obtained (preterm 21, term 30). The other 123 were preterm infants with RDS (N=59), healthy preterm infants without RDS (N=12), infants over 29 gestational weeks with suspected early-onset infection (N=35), and healthy term infants aged 2 to 5 days (N=17). Of the preterm and term infants with only cord blood samples available, 11 (preterm) and 29 (term) were included in two series (I, III). In Study I, 25, and in Study III, 15 healthy adults taking no medication, and with no clinical signs of infection, served as controls.

Main results: Neutrophil CD11b expression and plasma IL-8 concentration were higher in septic infants at the initial evaluation. Their sensitivity and specificity with the chosen cut-off value were high. In preterm infants with RDS, neutrophil CD11b expression was increased on the first day of life. The administration of dexamethasone inhibited phagocyte activation and systemic inflammation in preterm infants with RDS. Both preterm and term infants had a lower capacity to increase neutrophil CD11b expression upon *in-vitro* stimulation, whereas the ability to release BPI was lower only in preterm infants.

Conclusions: Neutrophil CD11b expression and plasma IL-8 serve as reliable markers for early onset sepsis in newborn infants born at or near term. In preterm infants, phagocyte activation and systemic inflammation play a role in the pathogenesis of RDS. Because inflammatory factors are important in the etiology of bronchopulmonary dysplasia (BPD), decreased phagocyte activation in the circulation may lead to attenuated pulmonary inflammation and to a reduced severity of BPD. The lower ability of PMN in newborn infants to release granule contents may, at least in part, explain the higher susceptibility of newborn infants to infections than adults.

1. INTRODUCTION

During the neonatal period, PMNs, monocytes, and natural killer (NK) cells play an important role in host defence. They are cellular components of the innate immune system, and thus, unlike T- and B-lymphocytes, prior exposure to foreign antigen(s) is not a prerequisite for their activation. Newborn infants show increased susceptibility to bacterial and fungal infections. The reason for this is considered to derive at least in part from an undeveloped immune system and defects in the innate defence (Ferrieri, 1990). Some components of the innate system, such as bacterial ingestion and killing, are fully functional at the time of birth (Shigeoka *et al*., 1979), while other features, like a limited bone marrow storage pool (Christensen and Rothstein, 1980; Christensen *et al*., 1982), denote immaturity of the host defence.

Among newborn infants, bacterial sepsis, causing major morbidity and mortality, especially in preterm infants (Philip, 1994; Beck-Sague *et al*., 1994), is one of the most common diagnostic challenges in neonatal care. The clinical signs of sepsis are variable and unspecific. The definite diagnosis, based on blood culture, is delayed, and highly sensitive and specific early markers of sepsis are lacking. Hence it is a common approach to begin the antimicrobial treatment on the basis of clinical suspicion of sepsis. As a result, antimicrobials are used excessively and, indeed, in most cases unnecessarily, when analyzed in retrospect. This practice renders the infants susceptible to potential side effects of the antimicrobials and, in addition, encourages the development and spread of drug-resistant bacteria, at present an increasing problem in hospitals worldwide. Evidently, early markers of sepsis are needed to identify those neonates who benefit from the antimicrobial therapy.

2. REVIEW OF THE LITERATURE

2. 1 INFLAMMATION

An inflammatory response is characterized by recognition of the site of injury by inflammatory cells, recruitment of subpopulations of leukocytes into the tissue, removal of the offending agent and cell debris, and repair of the site of injury. This complex physiologic process involves the interaction between cell surface, adhesion molecules, extracellular matrix, and soluble mediators such as cytokines, platelet activating factor, eicosanoids originating in cells, histamine, and mediators of plasma such as the complement system, the kinin sytem, the coagulation system, and the fibrinolytic system (Gahmberg *et al*., 1997; Delves and Roitt, 2000).

The immune responses are of two types: innate and adaptive. The innate (non-adaptive) immune system comprises humoral components such as the complement system, fibronectin, C-reactive protein, lactoferrin, and cytokines, and phagocytic cells such as polymorphonuclear neutrophils (PMN), monocytes, and macrophages. In the innate immunity, the response is similar on repeated exposure to a given pathogen because of unspecific recognition systems, yet depending on the amount of pathogens, their localization, and the developmental stage of the host. The adaptive immune response, mediated by lymphocytes is, in contrast, specific for a particular pathogen, improving with repeated exposure to the same pathogen (Delves and Roitt, 2000).

At the site of injury or inflammation, the first-line cells are phagocytes (Roitt and Male, 2001). The recruitment of phagocytes is preceded by their adhesion to vascular endothelium. The phagocyte-endothelial interaction is, however, a double-edged sword. Occasionally the response of the inflammatory defence mechanism to infection or injury is of a greater magnitude and more prolonged than is necessary, leading to a pathologic process and tissue injury.

2.1.1 Cells

Polymorphonuclear neutrophils

PMNs are key components of the innate immunity, and the most effective killing phagocytes of the host defence. They accumulate quickly and in vast numbers at sites of infection, where they phagocytize and destroy foreign material such as bacteria. In the bone marrow, colony-forming unit granulocyte-macrophage (CFU-GM) progenitor cells differentiate into PMNs and monocytes. The first PMN precursor, the myeloblast, produces daughter cells called myelocytes. Myelocytes do not proliferate, but require about a week to become fully differentiated PMNs. After leaving the bone marrow, mature PMNs circulate approximately 6 to 8 hours before infiltrating into tissue, where they live for an additional 24 hours without re-entering blood vessels (Fanaroff and Martin, 2002). Half of the PMNs in the blood stream are found in a marginating pool and half circulates in the peripheral blood (Cronkite, 1979).

Monocytes and macrophages

CFU-GM progenitor cells differentiate into promonocytes and then into circulating monocytes. After leaving the bone marrow, the newly formed mononuclear phagocytes circulate for 12 to 32 hours (Nichols and Bainton, 1973). Once they leave the circulation and migrate into tissues, they differentiate into macrophages. In the tissue they are in some circumstances able to live for months or even for years. The recruitment of mononuclear phagocytes occurs after 6 to 12 hours from the PMN influx (Fanaroff and Martin, 2002). Certain morphologic changes occur upon differentiation process into macrophages. The cell increases in diameter more than threefold and acquires more cytoplasmic granules and vacuoles. Cell surface receptors increase in number, and the phagocytic activity improves (Gordon *et al*., 1995).

Mononuclear phagocytes play central roles in inflammation, immune responses, and wound healing. They are able to eliminate microbes, remove senescent and transformed cells, and initiate antigen processing for initiation of specific immune response (Lasser, 1983).

Lymphocytes

Acquired immunity is characterized by cell-mediated and antibody-mediated responses. Lymphocytes, constituting about 20% of blood leukocytes in adults, are derived from lymphoid progenitors in the bone marrow and lymphoid organs. They are specialized in the recognition of antigens. The two main types of lymphocytes are B cells that may differentiate into antibody-producing plasma cells, and T-cells that differentiate in the thymus. T cells help B cells to make antibodies, kill virally infected cells, regulate the level of the immune response and stimulate the microbial and cytotoxic activity of other immune effector cells (Roitt and Male, 2001; Fanaroff and Martin, 2002). Each lymphocyte carries a surface receptor that is able to recognize a particular antigen.

2.1.2 Soluble mediators

Cytokines

Immunoglobulins produced by B-cells, complement proteins, and cytokines comprise the soluble mediators of inflammation. Cytokines, produced by monocytes, macrophages, and T-cells, are a group of small hormone-like signaling molecules that play regulatory roles in host defense and in normal and abnormal homeostatic mechanisms (Cohen and Cohen, 1996). Cytokines are proteins which are produced by diverse cell types and exert their function on a variety of cells. They suppress or enhance cellular proliferation, differentiation, activation and motility. The production of cytokines is usually transient and tightly regulated. The binding of a cytokine to its specific receptor begins a cascade that leads to induction or inhibition of trancription of a number of cytokine-regulated genes. Any cytokine may have many different biologic effects depending on the target cell. However, different cytokines may have similar effects (Roitt and Male, 2001).

TNFα and IL-1β

The proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) mediate the primary responses in inflammation (Tracey *et al*., 1988; Waage *et al*., 1988; Blackwell and Christman, 1996), contributing substantially to the host´s primary response to infection. They are produced in large quantities in macrophages, as a response to microbial products. Both produce a sepsis-like syndrome when injected into animals. In experimental settings, they are capable of inducing the same symptoms and the same level of severity of septic shock and organ dysfunction as does endotoxin (Weinberg *et al*, 1992).

Interleukin-8

IL-8 is one of the most powerful chemoattractive agents for neutrophils (Peveri *et al*., 1988). It is a member of C-X-C chemokines, which all have PMN selective chemotactic activity. Each C-X-C chemokine has the structure of one amino acid (X) separating two cysteine residues (C). IL-8 is secreted by activated monocytes (Yoshimura *et al*., 1987; Schröder *et al*., 1987), neutrophils (Bazzoni *et al*., 1991), endothelial cells (Gimbrone *et al*., 1989), fibroblasts (Strieter *et al*., 1989), lung epithelial cells (Standiford *et al*., 1990), and by mitogen-stimulated T lymphocytes (Schröder *et al*., 1988) all upon challenge with lipopolysaccharide (LPS), TNF-α, or IL-1. IL-8, secreted by endothelial cells, induces the rapid shedding of L-selectin and the up-regulation of CD11b/CD18 and the attachment and transmigration of neutrophils (Huber *et al*., 1991).

In inflammatory and infectious diseases, increased concentrations of the proinflammatory cytokines IL-6, TNF-α, and IL-8 are found in plasma (Hack *et al*., 1989; Damas *et al*., 1989; Van Zee *et al*., 1991; Waage *et al*., 1989; Damas *et al*., 1997), suggesting that saturation of specific binding sites has occurred or an equilibrium between receptor-bound and free cytokines has been reached. Of these cytokines, IL-8 is found in the bloodstream together with IL-6 after the peaks of TNF-α and IL-1-β concentrations are reached in animal or human endotoxemia models (Van Zee *et al*., 1991). In septic patients, plasma IL-8 has the best correlation with the severity of the illness (Damas *et al*., 1997). In addition to increased plasma levels, high levels of leukocyte-associated IL-8 have been found in patients with sepsis syndrome (Marie *et al*., 1997).

Accurate determination of IL-8 levels in body fluids with a highly sensitive enzyme-linked immunosorbent assay is possible for clinical samples (Ida *et al*., 1992).

MIP-1-α

A macrophage-derived cytokine macrophage inflammatory protein-1 (MIP-1) is an LPS-inducible heparin-binding protein, which comprises two discrete peptides, MIP-1 α and MIP-1 β, members of the CC or β chemokines (C referring to a cystein residue) (Keane and Streiter, 2000). These peptides possess overlapping proinflammatory activities, but also antagonistic immunologic effects (Fahey *et al*., 1992).

MIP-1- α expression can be induced in a variety of cell types including monocytes, macrophages, mast cells, Langerhans cells, fibroblasts, T-cells, and PMNs (Kasama *et al*., 1993). MIP-1-α is induced in macrophages by LPS, and in monocytes by their binding to endothelial cells (Lukacs *et*

al., 1994). It exerts predominant proinflammatory properties by inducing PMN chemokinesis, activating PMNs, and stimulating PMN superoxide production (Wolpe *et al*., 1988; Wolpe and Cerami, 1989), by inducing monocyte and lymphocyte migration, NK-cell chemotaxis, and modulating macrophage functions by inducing their TNF-α, IL-1, and IL-6 production (Fahey *et al*., 1992). In addition to inflammatory cells, activated endothelium releases proinflammatory cytokines, which can lead to high local cytokine concentrations on the endothelium. The significance of these concentrations, however, has not been elucidated.

2.1.3 Local inflammation

At sites where microbes penetrate cutaneous and mucosal barriers, tissue damage and complement activation cause the release of mediators of inflammation such as C5a, which is a fragment of complement, and a strong chemotactic peptide. These mediators diffuse to the adjoining venules, altering endothelial cell morphology and function at the site of infection. In the area of local inflammation, blood supply increases, bringing leukocytes and serum molecules to the affected site, and capillary permeability increases, allowing exudation of soluble mediators of inflammation such as antibodies, complements, and kininogens to the surroundings and the adjoining venules. In addition, leukocytes migrate out of venules into the surrounding tissue (Pittard *et al*., 1994; Asimakopoulos, 1999; Roitt and Male, 2001).

At sites of acute inflammation, the first leukocytes are PMNs, but at later stages, monocytes and lymphocytes predominate. The pathogen is destroyed by phagocytosis or cytotoxic reactions and by neutralization with specific antibodies. The repair of injured tissue begins during the early phases of inflammation, but is accomplished only after the noxious stimulus has been neutralized. Regeneration of parenchymal cells or scarring contributes to the repair process (Lasser, 1983; Roitt and Male, 2001; Delves and Roitt, 2000).

2.1.4 Systemic inflammation

Loss of any control of localized inflammation, or an exaggarated inflammatory response, may result in a systemic response contributing significantly to that morbidity and mortality often seen in sepsis, which is defined as the systemic response to infection (Bone *et al*., 1992). Systemic inflammation is characterized by the activation of inflammatory cells, the coagulation system, and the complement system within the circulation (Beal and Cerra, 1994). Primary responses in inflammation are mediated by proinflammatory cytokines such as TNF-α and IL-1, which induce secondary pro- and anti-inflammatory mediators such as IL-6, IL-8, and IL-10. Anti-inflammatory mediators such as IL-4, IL-10, IL-13 induce immunosuppression by inhibiting the activity of proinflammatory cytokines (Dinarello, 2000).

As the noxious stimulus is resolved, the proinflammatory response becomes downregulated. Sometimes, however, this homeostasis is not achieved, and the systemic inflammatory response may proceed to hypotension and circulatory collapse, and to the multiple organ dysfunction syndrome (Yao *et al*., 1998). The outcome of the disease in patients with systemic inflammation has been suggested to depend on the balance between the effects of pro- and anti-inflammatory cytokines (Dinarello, 2000).

The inflammatory response displays a high level of interindividual variation, which may be due to genomic variation leading to a high level of cytokine release during the inflammatory response and to poorer outcome (Stüber *et al*., 2001).

2.1.5 Phagocyte-endothelial cell interactions

Adhesion to the endothelium and transmigration

The first step in the process of emigration is the selectin-mediated reversible PMN tethering to the activated endothelium on postcapillary venules (Figure 1). Selectins are sugar-binding cell surface glycoproteins, of which L-selectin (CD62L) is constitutively expressed on the surface of leukocytes, and shed after cellular activation (Smith CW *et al*., 1991). E-selectin (CD62E) is expressed on activated endothelial cells, and P-selectin (CD62P) is expressed both on the endothelium and on platelets (Carlos and Harlan, 1994; Gahmberg *et al*., 1992).

Selectins are characterized by an amino-terminal calcium-dependent lectin domain, an epidermal growth factor-like domain, repeated C3/C4 binding protein-like domains, a transmembrane region, and a short cytoplasmic tail (Tedder *et al*., 1989; Tedder *et al*., 1990; Gonzáles-Amaro and Sánchez-Madrid, 1999).

Each of the three selectins recognizes certain sialylated and fucosylated oligosaccharides such as sialyl Lewis X. The main leukocyte glycoprotein ligand is P-selectin glycoprotein ligand-1, which serves also as a ligand for L- and E-selectins. E-selectin recognizes E-selectin ligand-1, the specific ligand on leukocytes. P- and E-selectin are upregulated by translocation from Weibel-Palade bodies and by *de novo* synthesis (McEver *et al*.,1989; Bevilacqua *et al*., 1989). Binding of selectins to their ligands on leukocytes causes leukocyte transition from the circulating state to a rolling state which slows the speed of the cells markedly (Lawrence and Springer, 1991).

In the second step of emigration, slowed leukocytes have the opportunity to respond to chemokines such as platelet activating factor (PAF), complement fragments, leukotriene B4, and IL-8, all synthesized by the endothelium. These chemokines are able to trigger particular populations of leukocytes that possess the appropriate chemokine receptors. After triggering, the cells become activated, resulting in surface upregulation of integrins (Figure 1).

In the third step, firm adhesion to the endothelium, primarily mediated by the integrin CD11b/CD18 (Mac-1, $\alpha_M\beta_{2,}$ CR3) is developed (Figure 1). Integrins are heterodimeric membrane glycoproteins consisting of α and β subunits (Patarroyo *et al*., 1985; Hynes, 1987; Kishimoto *et al*., 1989; Patarroyo *et al*., 1990), of which β_2 integrins share the common β-chain CD18, attached to CD11a (LFA-1), CD11b (Mac-1, $\alpha_M\beta_{2,}$ CR3), CD11c (p150,50) (Arnaout, 1990; Gahmberg, 1997), or CD11d (Van der Vieren *et al*., 1995). The most important in the adhesion process of PMNs being CD11b/CD18. CD11b/CD18 molecules are expressed at relatively low levels on the surface of resting neutrophils and monocytes.

Phagocyte activation is characterized by emergence of neo-epitopes on CD11b/CD18, which enable them to bind to their ligands, and by an increase in CD11b/CD18 complex density on neutrophils. Molecules of CD11b/CD18 translocate from the intracellular storage granules, *i.e*., secretory

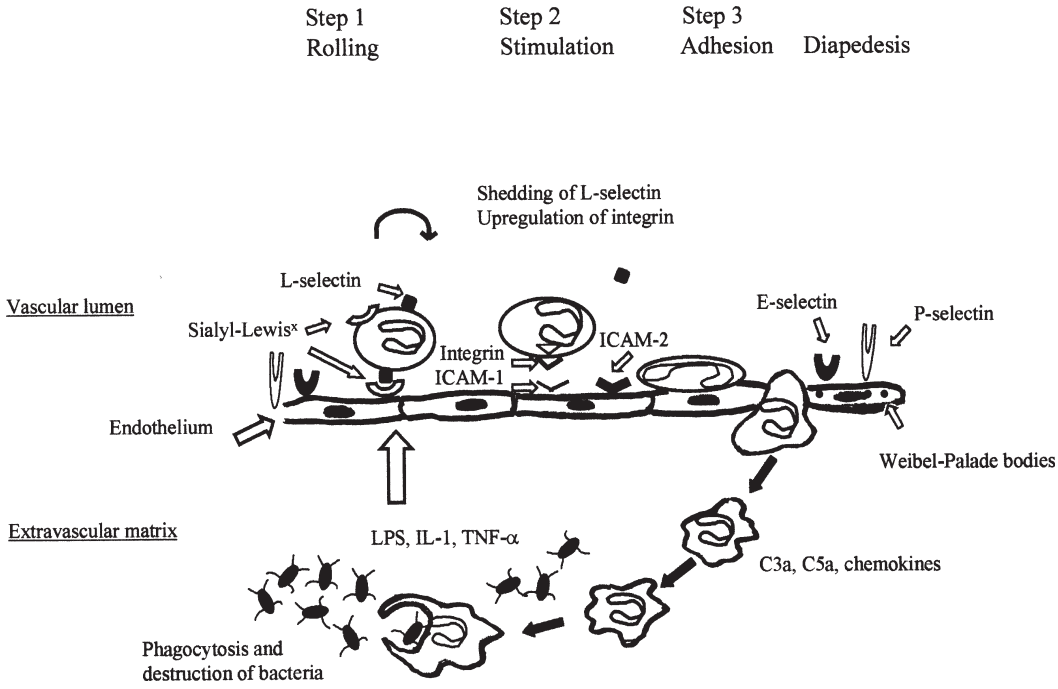


Figure 1. Adhesion of the polymorphonuclear leukocyte on the endothelium, transmigration, and chemotaxis.

vesicles and specific granules including gelatinase granules, to the cell surface (Calafat *et al*., 1993; Borregaard and Cowland, 1997). In the resting neutrophil, only 5% of the total cell content of CD11b/CD18 complexes is located on the cell surface, whereas 95% is located as membrane components of intracellular storage granules (Sengeløv *et al*., 1993a). After changes in conformation and clusterins, CD11b/CD18 is able to bind to its endothelial ligands intercellular adhesion molecule-1 (ICAM-1) and ICAM-2, members of the immunoglobulin superfamily (Xie *et al*., 1995; Henzen *et al*., 2000). CD11b/CD18 also binds to the red cell ICAM molecule (Bailly *et al*., 1995).

After the neutrophil´s firm attachment to the endothelium, its pseudopod is extended through the vessel at a junction between endothelial cells, accompanied by a flattening of the leukocyte against the vessel wall letting the leukocyte penetrate the wall by moving through interendothelial-cell junctions into the interstitium (Carlos and Harlan, 1994).

2.1.6 Chemotaxis

Chemotaxis is characterized by the directed migration of leukocytes across the wall of a venule and within the tissues along a chemokine concentration gradient. The population of cells which will cross the endothelium, and where they will move within the tissue, is determined by chemokines and adhesion molecules (Roitt and Male, 2001; Gahmberg et al., 1997). About 50 human chemokines and nearly 20 receptors have been identified and characterized since the discovery of interleukin-8, the first chemotactic cytokine (Baggiolini, 2001). Chemokines are secretory proteins produced either constitutively or after their induction by immigrating leukocytes and by virtually all other somatic cells in response to bacterial toxins and inflammatory cytokines such as IL-1, TNF-alpha and interferons. Chemokine activities are mediated through G-protein-coupled receptors, the largest known family of cell-surface receptors, which also mediate transmission of stimuli by hormones and various peptides (Iacovelli *et al.*, 1999; Baggiolini, 2001).

During interaction with chemokines, neutrophils undergo cytoskeletal rearrangements of shape resulting from the polymerization and de-polymerization of actin (Thelen *et al*., 1988), and the up-regulation of integrins on the cell surface prior to emigration (Springer, 1994).

Chemokines act locally rather than systemically; hence the levels in the body fluids reflect the disease process only weakly.

2.1.7 Degranulation

Neutrophils migrate rapidly into sites of infection, where they engulf and destroy bacteria either intracellularly or kill them extracellularly by mobilizing antibiotic proteins and peptides from cytoplasmic granules (Levy, 1996). Before they can be recognized by neutrophils, many microbes and particles need to be opsonized. The most important of these opsonins are complement fragments and immunoglobulins. For these opsonins, neutrophils have specific surface receptors which enable phagocytosis. Neutrophils contain numerous cytoplasmic granules that fuse with the plasma membrane of phagosomes and discharge toxic substances into the extracellular medium and into the phagosomes within seconds of ingestion.

The primary function of the microbicidal peptides, proteins, and enzymes is to defend the host against invading microbes. The neutrophil, on the other hand, is unable to differentiate between host and foreign antigens, which can lead to destruction of the surrounding tissue (Henson and Johnston Jr, 1987). Three types of granules exist in neutrophils: primary or azurophil granules, secondary or specific, and tertiary or gelatinase granules (Table 1). In addition, secretory vesicles, organelles formed by endocytosis without granule exocytosis, are found in neutrophils (Borregaard *et al*., 1987; Miller *et al*., 1987; Borregaard *et al*., 1990; Sengeløv *et al*., 1992).

Azurophil granules contain antibiotic and digestive proteins such as bactericidal permeability-increasing protein (BPI) and elastase, both of which have great importance during phagocytosis. Myeloperoxidase (MPO), a major component of the azurophilic granules of neutrophils and monocytes, is essential for the oxygen-dependent microbicidal effects of neutrophils. It is a heme protein which utilizes H_2O_2 generated by NADPH oxidase to make hypochlorous acid and other toxic agents (Nauseef *et al*., 1988).

The membranes of specific granules contain proteins such as lactoferrin, collagenase, gelatinase, and lysozyme. Moreover, of the total content of the adhesion molecule CD11b/CD18 in resting neutrophils, 5% is localized on the cell surface, 75% in the membrane components of specific granules, and 20% as membrane components of secretory vesicles (Sengeløv *et al*., 1993a).

The granules and secretory vesicles are mobilized in a hierarchic manner (Sengeløv *et al*., 1993b; Sengeløv *et al*., 1995). Gelatinase granules are exocytosed more readily than are specific granules, which in turn are more sensitive than azurophil granules (Dewald *et al*., 1982; Lew *et al*., 1986; Kjeldsen *et al*., 1992). Secretory vesicles are readily mobilized in response to a weak neutrophil stimulus that barely mobilizes the granules. Once mobilized, secretory vesicles are not reformed (Kobayashi and Robinson,1991).

Table 1. Contents of human neutrophil granules and secretory vesicles.

Azurophil Granules	Specific Granules	Gelatinase Granules	Secretory vesicles
Matrix: Elastase Myeloperoxidase Lysozyme Cathepsins Bactericidal permeability-increasing protein Defensins β-glucuronidase Proteinase-3 Azurocidin	Membrane: CD11b/CD18 Cytochrome b Matrix: Collagenase Gelatinase Lactoferrin Lysozyme fMLP-R	Membrane: CD11b/CD18 Cytochrome b Matrix: Gelatinase Lysozyme	Membrane: CD11b/CD18 Matrix: Plasma proteins

Bactericidal/permeability-increasing protein (BPI)

Antibacterial protein BPI, which is found in PMNs and eosinophils, selectively binds to the LPS on the outer membrane of gram-negative bacteria. This causes the immediate growth-arrest, irreversible damage, and death of these microbes. BPI also attenuates endotoxic effects of LPS and promotes phagocytosis of BPI-coated bacteria. It inhibits leukocyte activation, ameliorates endotoxic effects (Weiss and Olsson, 1987; Marra *et al*., 1990), inhibits LPS-induced neutrophil activation and cytokine secretion (Meszaros *et al*., 1993), and reduces tissue PMN deposition (Hansbrough *et al*., 1996).

BPI has been localized to azurophil granules, where its hydrophobic C-terminal end is believed to anchor it to the granule membrane. Data on isolated cells stimulated to release granule contents *in vitro* and of plasma derived from animals and humans with sepsis *in vivo* show that a portion of cellular BPI stores is released from stimulated PMNs (Weiss *et al*., 1982; Wong *et al*., 1995; Dentener *et al*., 1997; Froon *et al*., 1995; Froon *et al*., 1998).

2.1.8 Oxidative burst

In the oxygen-dependent mechanism of microbial killing, phagocytosing neutrophils undergo a burst of oxygen consumption caused by NADPH oxidase, a plasma membrane-associated enzyme in leukocytes. This catalyzes the production of superoxide (O_2^-) by the one-electron reduction of oxygen, with NADPH serving as the electron donor (Iyer, 1961; Robinson and Badwey, 1995):
$2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+$

The O_2^- which is generated serves as the starting material for the production of a range of other reactive oxidants, including halogens, free radicals, and singlet oxygen (Badwey and Karnovsky, 1980). Phagocytes use these oxidants to kill microbes, but these phagocytes can also cause damage to the surrounding tissue.

2.1.9 CD14

CD14 is a monocyte/macrophage cell-surface molecule acting as a binding receptor for bacterial LPS complexed with serum LPS-binding protein (LBP). LPS binding results in phagocyte activation by signaling via the members of the Toll-like receptors (TLRs). Of the TLRs, TLR2 plays a major role in Gram-positive bacterial recognition, whereas TLR4 has been shown to mediate LPS-induced signal transduction (Kirschning *et al*., 1998; Hoshino *et al*., 1999; Hallman *et al*., 2001).

TLRs signal activation of nuclear factor-kB (NF-kB), an important trancription factor in acute inflammation, which regulates inflammatory gene expression of various cytokines, enzymes, receptors, and adhesion molecules. This response is enhanced by the presence of CD14 and LBP.

2.1.10 C-reactive protein

C-reactive protein (CRP), an acute phase serum protein, is an important component of the innate immune system. CRP has been shown to provide protection from infection by activating the complement cascade (Nakayama *et al*., 1983; Horowitz *et al*., 1987; Szalai *et al*., 1996) and by interacting with the cells in the immune system by binding directly to F_C gamma receptors (Fcγ R) (Marnell *et al*., 1995; Bharadwaj *et al*., 1999). It may protect against the damaging inflammatory response to lipopolysaccharide and to host inflammatory mediators such as TNF, IL-1 and platelet activating factor (Xia and Samols, 1997; Chae *et al*., 2000).

Its concentration increases rapidly in response to infection, trauma, and inflammation. The CRP level is widely used as an indicator of infection and recovery from infection, although there are limitations in its use to discriminate infection from other causes of clinical illnesses. CRP production is stimulated primarily by IL-6 and IL-1, which are released in various types of acute inflammations. CRP may be considered as a marker of acute inflammation and tissue injury rather than as a specific marker of infection.

2.1.11 Suppression by glucocorticoids

Molecular mechanisms

Glucocorticoids (GCs) bind to glucocorticoid receptors (GCRs) in the cytoplasm which then dimerize and translocate to the nucleus. In the nucleus, GCs increase the transcription of glucocorticoid-responsive genes coding for anti-inflammatory proteins such as lipocortin-1, IL-10, IL-1 receptor antagonist, and neutral endopeptidase (Barnes, 1998).

GC is able to inhibit NF-κB. In addition, GCs can inhibit transcription of proinflammatory cytokines by direct binding of activated GCRs to glucocorticoid responsive elements in the promoter regions of responsive genes (Christman *et al*., 1998; Barnes, 1998).

In addition, GCs are able to change the chromatin structure (Johnson *et al*., 1979) by tightening the coiling of DNA and reducing the access of transcription factors to their binding sites, thereby suppressing gene expression.

Effects on inflammatory cells and on the endothelium

Well-known pharmacologic effects of GCs in humans are leukocytosis (Athens *et al*., 1961; Nakagawa *et al*., 1998), inhibition of leukocyte recruitment to inflammed areas (Zweiman *et al.*, 1976; Korompilias *et al*., 1996), retention of lymphocytes in the lymphatic circulation with shrinkage of peripheral lymph nodes, and promotion of microbial infection (Parrillo and Fauci, 1979).

GCs inhibit the release of inflammatory mediators and cytokines from alveolar macrophages *in vitro* (Fuller *et al.* , 1984; Linden and Brattsand, 1994). Systemic GC increases peripheral neutrophil counts and survival time by inhibiting their apoptosis (Cox, 1995).

2.2 PHAGOCYTE FUNCTIONAL DEFECTS

In the human fetus, PMN precursors and macrophages are first identified in the yolk-sac stage of primitive hematopoiesis (Yoder, 2000). By approximately 14 weeks of gestation, mature PMNs are identifiable in the fetal liver or bone marrow. In the normal fetus, in the second trimester, PMN counts are very low, averaging 0.24 to 0.35 x 10^{9}/L at 22 to 29 weeks (Forestier *et al*., 1986), and 0.77 x 10^{9}/L at 31 weeks to 8.53 x 10^{9}/L at term (Davies *et al.*, 1992). Thus, infants born prematurely have physiologic neutropenia.

Circulating monocytes appear in fetal blood after the age of five months of gestation. At 30 weeks´gestation, monocytes increase to 3 to 7% of the circulating blood cells. At birth and in the neonatal period, circulating monocyte count exceeds that of adults (Fanaroff and Martin, 2000).

In normal situations, labor results in neutrophilia, which is observed in the early neonatal period (Herson *et al.*, 1992). The number of circulating PMNs declines slowly by 72 hours to values that are seen in the neonatal period (Fanaroff and Martin, 2000). In addition, labor has been shown to increase neutrophil function or to have no effect on it (Herson *et al.*, 1992; Usmani *et al.*, 1993; Buonocore *et al.*, 1995). During sepsis, however, newborn infants frequently become neutropenic

because of their limited neutrophil storage pools in the bone marrow and their inability to increase stem cell profileration (Christensen and Rothstein, 1980; Christensen *et al.*, 1982). In addition, newborn infants born to mothers with hypertension have abnormally low blood neutrophil concentrations (Manroe *et al.*, 1979), due to decreased neutrophil production (Koenig and Christensen, 1989). Total neutrophil mass and the capacity to increase progenitor proliferation in preterm infants are even lower (Carr *et al.*, 1992a).

In newborn infants, neutrophils express granulocyte colony-stimulating factor (G-CSF) receptor less than in adults (Gessler *et al.*, 1999). This receptor mediates the effects of G-CSF such as neutrophilia and enhancement of effector functions of PMNs.

In any newborn infant, PMN accumulate poorly at the sites of infection as a result of chemotactic deficiencies (Andersson *et al.*, 1981; Bektas *et al.*, 1990; Santos and Davidson, 1993). In preterm infants, chemotactic maturation begins after 2 to 3 weeks of life, proceeding slowly. In term infants, normal chemotactic function is established by the age of 2 weeks, whereas in preterm infants, chemotactic motility remains impaired for at least 3 weeks (Eisenfeld *et al.*, 1990; Usmani *et al.*, 1991). In addition, in newborn infants, monocyte influx into inflammatory sites is impaired most likely due to the delayed and attenuated monocyte chemotactic activity (Fanaroff and Martin, 2000).

Phagocytosis and microbicidal activity of phagocytes of healthy term newborn infants appear to be mature (Speer *et al*., 1986; Speer *et al*., 1988; Conly and Speert, 1991) although in preterm infants and in septic or stressed infants, the neutrophil respiratory burst activity, phagocytosing capacity, or killing capacity are significantly depressed (Gahr *et al*., 1985; Bortolussi *et al*., 1993; Falconer *et al*., 1995; Drossou *et al*., 1997).

Upon chemotactic stimulation, neonatal PMNs generate less of the filamentous actin and microtubules which are essential for locomotion (Sacchi *et al*., 1987; Anderson *et al*., 1984).

In newborn infants, the expression of neutrophil Fcγ-receptors, which recognize the Fc portion of IgG and bind to immune complexes or IgG-opsonised particles, is lower than that in adults (Smith *et al*., 1990).

Reports exist, in term newborn infants, of diminished levels of L-selectin at the surface of resting neutrophils and decreased downregulation of L-selectin compared with that of adults (Anderson *et al*., 1991; Török *et al*., 1993; Koenig *et al*., 1996). Other reports have shown normal adult levels of L-selectin on the surface of resting and stimulated fetal PMNs (Smith and Tabsh, 1993). The content of CD11b/CD18 in PMNs from newborn infants is lower than that in adults (Abughali *et al*., 1994; McEvoy *et al*., 1996). They adhere to and migrate through endothelium inefficiently (Andersson *et al*., 1990). That PMNs from newborn infants express less membrane and cytoplasmic CD11b/CD18 renders these PMNs deficient in transmigrating through endothelium and in bactericidal activity. In leukocyte adhesion deficiency syndrome, the deficient cell surface expression of CD11b/CD18 renders these patients to severe and recurrent bacterial infections (Arnaout *et al.*, 1990).

2.3 PERINATAL INFECTIONS

Transmission of infections from the biologic mother to her offspring which is known as perinatal infection, is still a major problem in neonatal care. In the newborn infant, the symptoms are non-specific and indistuinguishable from those of various other diseases in the newborn period, which makes diagnosis difficult.

2.3.1 Intrauterine infections

Intrauterine infection (IUI) is an infection of the normally sterile content of the uterus. Intra-amniotic infection (IAI), which is a subset of intrauterine infections, refers to a positive amniotic fluid bacterial staining or culture. IAI is clinically diagnosed in 0.5-10.5% of pregnancies (Hillier *et al*., 1988; Soper *et al*., 1989). Extra-amniotic infection, characterized by histological inflammation of the chorion and amnion, with a higher frequency in preterm pregnancies (Hillier *et al*., 1988; Hansen *et al*., 2000), can spread and result in microbial invasion of the amniotic fluid, intra-amniotic infection. IUI contributes significantly to the pathogenesis of preterm labor, likely by the release of proinflammatory cytokines that stimulate uterogenic agents such as prostaglandin E_2 and F2 biosynthesis by the decidua or amnion and thus initiate labor (Mitchell *et al*., 1991).

In IUI, several studies have shown increased concentrations of proinflammatory cytokines in amniotic fluid (Romero *et al*., 1990; Hillier *et al*., 1993; Romero *et al*., 1993; Negishi *et al*., 1996; Arntzen *et al*., 1998), or in cord blood (Lencki *et al*., 1994; Salafia *et al*., 1997). Of these cytokines, IL-1β, IL-6, and TNF-α are released by the PMNs infiltrating the membranes in chorioamnionitis (Steinborn *et al*., 1999).

The risk for IAI increases greatly after rupture of the fetal membranes. Premature rupture of the membranes (PROM), rupture before the onset of labor, is present in approximately 10% of all pregnancies (Alexander and Cox, 1996). PROM and preterm PROM (pPROM), membrane rupture before 37 completed gestational weeks, are associated with complications such as preterm labor, neonatal sepsis, and intrauterine infection. The risk of chorioamnionitis is markedly higher in patients with PROM and preterm labor compared with patients with only preterm labor (Guzick and Winn, 1985; Seo *et al*., 1992).

Fetal immune response

Current evidence suggests that in intrauterine infection the fetus involved may become infected and develop fetal immune response (FIR), possibly before the mother does (Romero *et al*., 1998). FIR can be defined as an increased plasma IL-6 concentration in the fetus, and is associated with increased perinatal morbidity (Gomez *et al*., 1998). In adults, an elevated IL-6 concentration of this magnitude may lead to an acute-phase reaction and systemic inflammatory response syndrome, and to tissue damage (Rangel-Frausto *et al*., 1995). In cord blood, the elevated IL-6 concentration is suggested to be a consequence of microorganisms in the amniotic cavity or maternal compartment, organisms that reach the fetus and stimulate the fetoplacental immune system with their cytokine production (Gomez *et al*., 1997).

2.3.2 Sepsis

Etiology and risk factors

Neonatal sepsis is divided into very early-onset, early onset, and late onset. The symptoms begin less than 12 hours from birth (very early onset sepsis), at the age of 12 to 72 hours (early-onset sepsis), or after 72 hours of age (late-onset sepsis). Maternal risk factors for infection are present almost always at very early onset, and frequently in early onset sepsis, but they are unrelated to late onset sepsis (Remington and Klein, 1995).

The incidence of early-onset sepsis is 1 to 10 cases per 1000 livebirths with a mortality rate of 15 to 50% (Wilson, 1990; Greenough, 1996). In very low-birth-weight neonates the incidence is increased approximately tenfold (Stoll *et al*., 1996a). In those preterm infants who need longer hospitalization, the incidence of nosocomial late onset bacterial infections is reported to be as high as 11 to 25% (Wilson, 1990; Remington and Klein, 1995; Stoll *et al*., 1996b; Greenough, 1996).

The fetal environment is normally sterile until the onset of labor and delivery. After rupture of the membranes, the infant becomes colonized with micro-organisms from the maternal genital tract. The micro-organisms recognized to have significant association with neonatal infections are group B *streptococci*, coagulase-negative *staphylococci*, group A *streptococci*, *Hemophilus influenzae*, *E. coli*. (Kaftan, 1998). Risk factors for neonatal sepsis are chorioamnionitis or endometritis, preterm delivery, group B *streptococci* colonization, preterm rupture of the membranes, and a prolonged duration of internal monitoring (Yancey *et al*., 1996).

Diagnosis and differential diagnosis

To detect neonatal sepsis early in its course is difficult, because the signs of infection are similar to those in various noninfectious diseases such as transitory tachypnea of the neonate, respiratory distress, meconium aspiration syndrome, or congenital heart defect. Clinical signs of sepsis can be divided into five categories depending on the symptoms from various organ systems: temperature instability (hypothermia, hyperthermia); respiratory distress (grunting, intercostal retractions, apnea, cyanosis); cardiovascular (tachycardia, bradycardia, poor perfusion, shock); neurologic (hypotonia, lethargy); gastrointestinal (feeding intolerance, abdominal distension).

The biological parameters currently used in the diagnosis of neonatal sepsis are blood culture, cerebro-spinal fluid culture, urine antigen test, leukocyte indices, and plasma level of CRP.

A positive blood culture with clinical signs of bacterial sepsis is widely accepted as a proof of bacterial infection. However, a negative result does not rule out sepsis; newborn infants may have clinical sepsis with a negative blood culture, yet typical symptoms and laboratory markers of infection (*e.g.* elevated immature leukocyte counts, leukopenia, increased CRP).

Meningitis in infants less than 72 hours of age is rare (Wiswell *et al*., 1995). Lumbar puncture for cerebro-spinal fluid culture for the detection of meningitis has been recommended for those with positive blood culture, and for those with two or more signs of sepsis (Kaftan, 1998).

The accuracy of urinary group B streptococcal antigen test has been questioned because of its high false-positive rates (Harris *et al*., 1989; Sanchez *et al*., 1990).

White blood cell count, band form count and related ratios have served as diagnostic tools for neonatal infections. The specificity and sensitivity of these tests, however, are insufficient to serve as the only markers for sepsis (Da Silva *et al*., 1995).

CRP is an acute-phase protein synthesized by the liver within 6 to 8 hours after an inflammatory process or tissue injury (Pepys, 1981). CRP alone is of limited value at the onset of symptoms, since especially in the newborn infant, there is delay in its elevation and lack of sensitivity (Da Silva *et al*., 1995; Mathers and Pohlandt, 1987). Serial CRP levels, on the other hand, are useful in the diagnostic evaluation of newborn infants with suspected sepsis (Ehl *et al*., 1997; Benitz *et al*., 1998).

Many attempts have been made to find a marker for bacterial infection that is both sensitive and specific. Among these markers tested are various cytokines in plasma or their gene expression in cells (Gessler *et al*., 1993; Roman *et al*., 1993; De Bont *et al*., 1994; Buck *et al*., 1994; Özdemir *et al*., 1994; Harris *et al*., 1994; De Bont *et al*., 1995; Kennon *et al*., 1996; Messer *et a*l., 1996; Panero *et al*., 1997; Berner *et al*., 1998; Doellner *et al*., 1998a; Kuster *et al*., 1998; Franz *et al*., 1999; Berner *et al*., 2000), their soluble receptors (Spear *et al*., 1995; Doellner *et al*., 1998b), soluble adhesion molecules (Lehrnbecher *et al*., 1996; Austgulen *et al*., 1997), indices of complement activation (Peakman *et al*., 1992; Zilow *et al*.,1993), plasma PMN-elastase or elastase-alpha-1 proteinase inhibitor (Tsaka *et al*., 1990; Salzer *et al*.,1993), urinary nitric oxide (Ergenekon *et al*., 2000), plasma procalcitonin (Gendrel *et al*., 1996; Monneret *et al*., 1997; Chiesa *et al*., 1998), and neutrophil CD11b expression (Weirich *et al*., 1998).

Treatment

Early diagnosis and initiation of antibiotic therapy with appropriate management of metabolic and cardiovascular disturbancies, and respiratory problems, can greatly affect the outcome of the septic infants. It is therefore imperative that empiric antimicrobial therapy is instituted immediatedly after obtaining culture samples. The choice of empiric therapy depends on the timing and setting of the disease, *e.g.* whether it is early-onset, late-onset, or of nosocomial type. The chosen antimicrobial therapy is also based on the micro-organisms most frequently encountered, the susceptibility profiles for these organisms, the site of the suspected infection, the penetration of the specific antibiotic to that site, and the safety of the antibiotic. In early-onset neonatal infection the antimicrobials should cover GBS, *E. coli*, and other gram-negative enteric bacilli, and *Listeria monocytogenes*. Therefore a combination of ampicillin and aminoglycoside is widely recommended.

2.4 Inflammation-associated diseases

2.4.1 Respiratory distress syndrome

In lung injuries, PMNs disappear from the peripheral blood into the pulmonary circulation, and subsequently migrate into the lung parenchyma. In preterm infants with respiratory distress, a low concentration of mature circulating PMN was associated with more severe RDS during the first postnatal week (Ferreira *et al*., 2000). In addition, in the lungs of preterm infants with RDS, an

early inflammatory reaction with neutrophils and macrophages is found (Merritt *et al*., 1981a; Merritt *et al*., 1981b; Merritt *et al*., 1983; Speer *et al*., 1993; Murch *et al*., 1996a; Murch *et al*., 1996b).

2.4.2 Bronchopulmonary dysplasia

BPD, a chronic lung disease in prematurely born infants, is defined as a need for supplemental oxygen and radiologic abnormalities at the age of 36 gestational weeks (Shennan, 1988).
Prematurity, barotrauma, and oxygen toxicity are important factors in the pathogenesis of BPD (Bancalari and Sosenko, 1990). However, fetal systemic inflammation and chorioamnionitis of the mother, in addition with postnatal infections, and pulmonary inflammation, have been shown to provoke early pulmonary inflammation and the development of BPD (Merritt *et al*., 1981a; Merritt *et al*., 1981b; Speer *et al*., 1993; Groneck *et al*., 1994; Kotecha *et al*., 1996; Groneck *et al*., 1996; Watterberg *et al*., 1996; Yoon *et al*., 1999; Groneck *et al*., 2001; Schmidt *et al*., 2001).

2.4.3 Necrotizing enterocolitis

Necrotizing enterocolitis (NEC), an intestinal emergency in mainly preterm newborn infants, is multifactorial in etiology. Risk factors for NEC that have been identified are prematurity, enteral feeding, bowel ischaemia, and infectious agents. Immature human enterocytes in vitro react with excessive pro-inflammatory cytokine production after inflammatory stimulation (Nanthakumar *et al*., 2000), and inflammatory cytokines seem to be involved in neutrophil recruitment and augmentation of the inflammatory response in neonatal intestine in infants with NEC (Viscardi *et al*., 1997).

2.4.4 Intraventricular hemorrhage, periventricular leukomalacia and cerebral palsy

Prenatal IUI appears to increase the risk of neonatal brain damage such as intraventricular hemorrhage (IVH), periventricular hemorrhagic infarction, periventricular leukomalacia (PVL), and cerebral plasy (CP). IVH is found 3 to 4 times more often in preterm infants born to mothers with chorioamnionitis than to mothers without infections (Morales, 1987; Yoon *et al*, 1995; Salafia *et al*, 1995; Alexander *et al*., 1998; The Developmental Epidemiology Network Investigators, 1998). White matter damage, *i.e*. PVL, and CP are associated with IUI as well (Murphy *et al.*, 1995; Dammann and Leviton, 1997; Yoon *et al.*, 1997a; Verma *et al*., 1997; Alexander *et al.*, 1998; Dammann et al., 1998; O´Shea *et al*., 1998). More than half of the very preterm infants with PVL lesions in the brains later develop cerebral palsy (Leviton and Paneth, 1990), of which spastic diplegia is the most prevalent (Pharoah *et al*., 1996).

Cytokines are suggested to constitute the link between brain lesions and IUI by crossing the blood brain barrier and damaging the white matter directly, by causing IVH, or by inducing further local cytokine production by microglial cells and astrocytes (Hopkins and Rothwell, 1995). Increased concentrations of proinflammatory cytokines are found in brain lesions in infants with periventricular leukomalacia (Yoon *et al*., 1997b). In addition, these cytokines are found in amniotic fluid (Figueroa *et al*., 1996; Yoon *et al*., 2000), in cord blood (Yoon *et al*., 1996), and in neonatal blood (Nelson *et al*., 1998) in preterm infants who later develop whiter matter lesions or CP.

3. AIMS OF THE STUDY

1. To investigate neutrophil CD11b/CD18 as a marker of early-onset sepsis, and a marker of systemic inflammation in newborn infants (I, II)
2. To study the effect of early-onset dexamethasone treatment on systemic inflammation in preterm infants with respiratory distress syndrome (IV)
3. To study the ability of neutrophils from newborn infants to degranulate proteins from the intracellular granules upon stimulation (I, III).

4. MATERIALS AND METHODS

4.1 PATIENTS

The studies were carried out at the Hospital for Children and Adolescents (I, II, III, IV), and in the Department of Obstetrics and Gynecology (II, III), at Helsinki University Central Hospital. The laboratory assays were performed at the Department of Bacteriology and Immunology of the Haartman Institute, University of Helsinki, and in the Department of Pathology, University of Turku. Each study protocol was approved by the institutional review boards in the participating hospitals, and informed consent was obtained from the parents.

The entire project comprised 174 newborn infants. From 51 of these infants, only cord blood was obtained (preterm 21, term 30). The other 123 were preterm infants with RDS (N=59), healthy preterm infants without RDS (N=12), infants over 29 gestational weeks with suspected early-onset infection (N=35), and healthy term infants aged 2 to 5 days (N=17) (Table 2). Of the preterm and term infants with only cord blood samples available, 11 (preterm) and 29 (term) were included in two series (I, III).

In Study I, 25, and in Study III, 15 healthy adults taking no medication, and with no clinical signs of infection, served as controls.

If any preterm infants with RDS had arterial alveolar oxygenation ratio below 0.22, surfactant treatment (Curosurf®, 100mg/kg, Chiesi Farmaceutici SPA, Parma, Italy) was started. RDS was diagnosed on the basis of clinical signs and radiologic findings.

Inclusion criteria in Study II were the presence of at least one clinical sign suggesting infection at the age of 0 to 48 hours, and a blood sample for bacterial culture having been requested by the clinician. Clinical signs were divided into five categories: temperature instability (hypothermia, hyperthermia); respiratory distress (grunting, intercostal retractions, apnea, cyanosis); cardiovascular (tachycardia, bradycardia, poor perfusion, shock); neurologic (hypotonia, lethargy); and gastrointestinal (feeding intolerance, abdominal distension). Sepsis was defined as a systemic response to infection with typical clinical signs, increased CRP concentration level and/or maternal risk factors for infection. Possible infection group comprised infants with non-infectious diagnoses in whom infection could not be ruled out.

In Study IV, infants with respiratory distress were randomized to receive dexamethasone (DEX group) from the first postnatal day, or to serve as controls (CONTROL group). Dexamethasone,

given as a four-day course, was started at the age of 12 to 24 hours at a dose of 0.5 mg/kg/day for 2 days and 0.25 mg/kg/day for the subsequent 2 days.

Table 2. Characteristics of the subjects.

	Study						
	I		II		III	IV	
Subjects	RDS+	RDS-	C. sepsis[1]	P. sepsis[2]	C.sepsis[1]	Dexa +	Dexa -
Preterm/term infants (N)	29/0	12/0	0/22	0/13	0/11	15/0	15/0
Gestational age[3] (wks)	27.8 (1.8)	33.3 (2.2)	37.6 (3.8)	37.4 (3.0)	38.0 (4.0)	29.1 (1.2)	29.2 (1.4)
Birth weight (g)	979 (263)	2125 (385)	2979 (1007)	3150 (874)	3279 (1109)	1223 (156)	1250 (148)
Preterm/Term cord (N)	11/30		21/29				

[1]Clinical sepsis; defined as positive blood culture, clinical symptoms consistent with sepsis without non-infectious disorders, and/or increased CRP concentration in plasma.
[2]Possible sepsis; bacterial infection impossible to exclude, non-infectious disorder(s) present
[3]Mean (SD)

4.2 BLOOD SAMPLING

The blood samples were drawn from umbilical veins (cord blood samples), from arterial cannulas, from femoral arteries or veins, or from cubital veins (adult controls) with pyrogen-free dry syringes (Ribbon Pack, Terumo Europe, Leuven, Belgium or B. Braun, Melsungen AG, Melsungen, Germany). Each sample of blood was added to a pyrogen-free tube containing citrate phosphate dextrose (Baxter Health Care Ltd., Norfolk, England; 0.14 mL/mL blood). The blood was immediately cooled to 0 °C in an ice-water bath to minimize neutrophil activation *ex vivo* (Repo *et al*., 1993; Repo *et al*., 1995) (I, II, III, IV), or kept in a 37°C water-bath for stimulation assays (I, III).

4.3 DETERMINATION OF CD11b/CD18, CD14, AND L-SELECTIN

4.3.1 Stimulation assays

Aliquots of a stock solution of formyl-methionyl leucyl-phenylalanine (FMLP) (Sigma Chemical Co., St. Louis, MO, USA) dissolved in dimethylsulphoxide at a concentration of $2x10^{-4}$ M were

stored at -20°C. The working solution was 10^{-6} M FMLP in phosphate buffer solution (PBS) at 37°C. An aliquot of working solution (2.5ml) was added to a 25 ml aliquot of blood sample kept at 37°C and further incubated at 37°C for 10 min (I,III). To study the ability of PMN to release BPI and MPO (III), two portions of 1 mL blood were anticoagulated and rapidly placed into a 37°C water-bath and immediately incubated with phorbol myristate acetate (PMA, final concentration 500 ng/mL). After incubation, the samples were kept at 0°C until stained for flow cytometry (I, III), or within 30 minutes the plasma was separated by centrifugation at 4°C and stored at -70°C until analysis (III, IV).

4.3.2 Cell labeling

Neutrophils (I,II,III,IV) in 25-µl aliquots of whole blood were labeled with saturating concentrations of the R-phycoerythrin (RPE) conjugate of mouse anti-CD11b IgG1 antibody (I, II, III, IV), and with the fluorescein isothiocyanate (FITC) conjugate of mouse anti-CD62L IgG2b antibody (IV) and their control antibodies. Monocytes, also in 25-µl aliquots, were labeled with the FITC conjugate of mouse anti-CD11b IgG1 antibody and the RPE conjugate of mouse anti-CD14 IGg2a antibody and their corresponding antibodies (IV). After the labeling, contaminating erythrocytes were lysed by addition of 2 mL of a 1/10 diluted ice-cold FACS lysing solution (Becton Dickinson, San Jose, CA, USA). After a 3-min incubation on ice, the leukocytes were centrifuged, and a second incubation with 2 mL of FACS lysing solution was performed for five minutes at room temperature. After centrifugation, leukocytes were resuspended in 1% formalin at 0°C.

4.3.3 Flow cytometry

FACScan flow cytometry (Becton Dickinson) and CellQuest analysis software (Becton Dickinson) were used for the acquisition and analysis of the data. Neutrophils were identified on the basis of their light-scattering properties, and monocytes on the basis of their CD14 positivity. For each sample, 5000 events were recorded. The expression of CD11b/CD18, L-selectin, and CD14 is reported in relative fluorescence units (RFU), *i.e.*, as the mean channel of the positive fluorescing cell population.

4.4 DETERMINATION OF SOLUBLE MEDIATORS

4.4.1 Interleukin-8

An IL-8 enzyme-linked immunosorbent assay kit (Quantikine, R&D Systems, Minneapolis, MN, USA) was used with the plasma samples in a blinded fashion. The detection limit of the assay, as indicated by the manufacturer, was 10 pg/mL. All samples were run in duplicate.

4.4.2 Plasma Bactericidal/Permeability-Increasing Protein

Plasma BPI was measured by time-resolved fluoroimmunoassay, and the amount of BPI detected in the supernatant is expressed as the BPI/PMN ratio (ng/10^6 PMN). All samples were run in duplicate.

4.4.3 Plasma Myeloperoxidase

Extracellular immunoreactive MPO was quantitated by use of commercial ELISA (R&D Systems, Abingdon, Oxford, UK). The amount of MPO detected in the supernatant is expressed as the MPO/PMN ratio (ng/10^6 PMN). The detection limit of the assay, as indicated by the manufacturer, was 20 mg/mL. All samples were run in duplicate.

4.4.4 Plasma Macrophage Inflammatory Protein 1-alpha

Extracellular immunoreactive MIP-1-α was quantitated by the use of commercial ELISA for MIP-1-α (R&D Systems, Minneapolis, MN, USA). The detection limit of the assay, as indicated by the manufacturer, was 20 ng/mL. All samples were run in duplicate.

4.5 DATA ANALYSIS

In Study II, statistical comparison between the groups (sepsis group, possible-infection group, and neonate control group) was performed with the Jonckheere-Terpstra test for ordered alternatives (Siegel, 1988), p-values being calculated by the Monte-Carlo method. Sensitivity and specificity, and their 95% confidence interval (CI) values were calculated for CD11b/CD18 expression, IL-8, and CRP. Receiver-operating characteristic (ROC) curves were used for the determination of thresholds for the sepsis group *vs.* healthy neonate group. The relationship between CD11b/CD18 and IL-8 was determined with the Spearman rank correlation test. The correlation coefficient (r) and its 95% CI are presented.

In Study I, statistical comparisons between groups were made by Kruskal-Wallis analysis of variance (ANOVA), t-test, or Welch´s test. Post hoc multiple comparisons were made by the Tukey or Tamhane´s T2 method. Significance of repeated measures was tested by repeated-measures analysis of variance. The normality of variables was evaluated by Shapiro-Wilk statistics. The most relevant descriptive values were expressed with a 95% confidence interval (95% CI). Correlation was estimated by Spearman´s coefficient method. The α level was set at 0.05 for all tests.

In Study III, the significance of difference between the groups was determined by Kruskal-Wallis analysis of variance (ANOVA). Two-group comparisons were done by the Mann-Whitney U-test, and the p-values were corrected by use of the Bonferroni test, if not otherwise indicated. Results are expressed as medians (range). To examine the correlation between PMN count and BPI, Spearman´s rank correlation was used.

In Study IV, statistical comparisons between the study groups were done by the Mann-Whitney U-test. Probabilities were regarded as statistically significant at the 0.05 level.

5. RESULTS

5.1 SEPSIS (II, III)

5.1.1. Neutrophil CD11b/CD18 and Interleukin-8 (II)

The sepsis group comprised 22 neonates, the possible-infection group 13 neonates, and the control group 12 healthy term neonates. Four neonates were blood-culture positive, two for group B *streptococci*, one for *E. coli*, and one for coagulase-negative *staphylococci*. No significant differences existed in mean gestational age or birth weight between the groups. One of the four infants excluded from the data analysis died of *Streptococcus agalactiae* sepsis at the age of 72 hours. The non-infective diagnoses of the infants in the possible-infection group were respiratory distress syndrome, maladaptation, anemia, interrupted aortic arch, and aspiration syndrome.

The levels of CD11b/CD18 expression on neutrophils correlated positively with plasma concentrations of IL-8 (Figure 2, r=0.82 [95 % CI 0.70 to 0.90]). No associations existed between birth weight or gestational age and levels of IL-8, CD11b/CD18 expression, or CRP. Visual analysis of the data indicates that the values of 12 healthy control neonates fell in the lower left quartile, whereas the values of 19 of the 22 neonates with sepsis fell in the upper right quartile (Figure 2). Of the three septic neonates outside the upper right quartile, the first had a high a CD11b expression level (246 RFU), low IL-8 level (17 pg/mL), and high CRP level (84 mg/L); with no risk factors for infection. The second had marginally elevated levels of CD11b/CD18 (176 RFU) and IL-8 (51 pg/mL); the mother had been treated with antimicrobial chemotherapy for clinical chorioamnionitis. The third´s CD11b/CD18 expression was 178 RFU and IL-8 concentration was 24 pg/mL; this mother had fever and symptoms of acute respiratory infection at delivery.

The majority of the values for the possible-infection group fell in the lower left quartile and none in the right upper quartile (Figure 2). The highest level of CD11b/CD18 expression (204 RFU) and IL-8 (92 pg/mL) in this group occurred in neonates whose mothers had clinical chorioamnionitis and were treated with antimicrobials. The three neonates with the lowest CD11b/CD18 expression levels (86, 93, 97 RFU) had diagnoses of anemia, maladaptation, and transitory tachypnea, respectively, but had no risk factors for infection. Their clinical signs resolved within 24 hours.

In the sepsis group, four neonates had high CD11b/CD18 expression levels (211, 255, 303, and 365 RFU, respectively), high IL-8 levels (126, 200, 62, and 107 pg/mL, respectively), and low CRP levels which did not increase during the follow-up of 3 to 4 days (range of the peak levels 5-8 mg/L). Their clinical signs persisted for over 24 hours, and antimicrobial treatment was continued for 7 days or more.

Levels of CD11b/CD18 expression, plasma IL-8 concentrations, and peak CRP concentrations all increased in the order: sepsis group > possible-infection group > healthy neonates (Table 3; p for monotonic trend <0.001). Peak CRP correlated with CD11b/CD18 expression (r=0.62 [95% CI 0.38 to 0.78]) and with IL-8 (r=0.58 [95% CI 0.33 to 0.76]).

ROC analysis served for determination of the best threshold for the sepsis group *vs.* healthy neonates. CD11b/CD18 expression, IL-8 concentration, and the peak CRP concentration had sensitivities of 1.00, 0.91, and 0.82, respectively, and each marker had a specificity of 1.00 (Table 4).

Table 3. Neutrophil CD11b/CD18 Expression Levels, Plasma IL-8 Concentrations, and Serum C-Reactive Protein (CRP) Concentrations.

Marker	Sepsis	Possible infection	Healthy neonates	p[1] between groups
	N=22	N=13	N=12	
	Median (range)	Median (range)	Median (range)	
CD11b/CD18 (RFU)	288 (176-519)	138 (86-204)	112 (76-145)	<0.001
IL-8 (pg/mL)	94 (17-9576)	24 (7-200)	17 (0.4-38)	<0.001
Peak CRP (mg/L)	48 (5-188)	11 (5-44)	6 (5-8)	0.002

[1]Jonckheere-Terpstra test for ordered alternatives

Table 4. Cut off Points, Sensitivity, and Specificity of the Markers in Diagnosis of Sepsis.

Marker	Cut off Point	Sensitivity (95% CI)	Specificity (95% CI)
CD11b/CD18 (RFU)	≥150	1.00 (0.87 to 1.00)	1.00 (CI 0.78 to 1.00)
IL-8 (pg/mL)	≥50	0.91 (0.73 to 0.98)	1.00 (0.78 to 1.00)
Peak CRP (mg/L)	>10	0.82 (CI 0.62 to 0.94)	1.00 (CI 0.78 to 1.00)

CI, confidence interval; RFU, relative fluorescence units

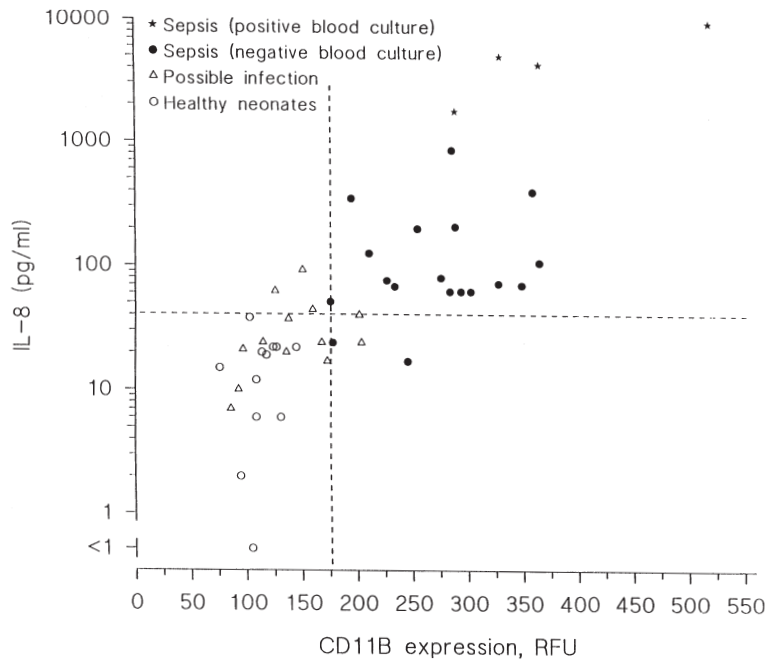


Figure 2. Correlation between neutrophil CD11b/CD18 expression level and IL-8 concentration. Dotted lines denote median values for the whole study population. RFU indicates relative fluorescence unit.

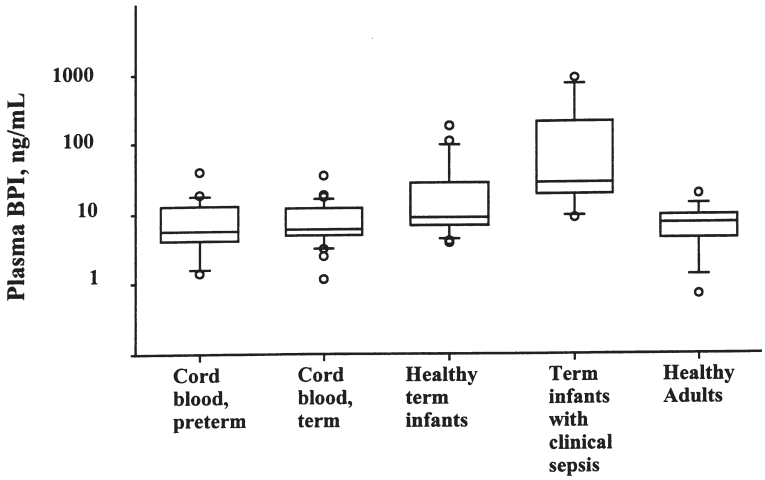


Figure 3. Box and whiskers-plot of BPI concentrations in plasma. The box indicates 75th and 25th percentiles with the central line the median. Whiskers represent the range with outliers indicated by open circles. Cord samples of preterm infants (n=21) and term infants (n=29), healthy term infants aged 2 to 5 days (n=17), term infants with clinical sepsis (n=11), healthy adults (n=15); p=0.014 between the groups (ANOVA); sepsis *vs.* healthy term infants, p=0.014 (without Bonferroni correction for multiple comparisons).

5.1.2. Bactericidal/Permeability-Increasing Protein (III)

The concentration of BPI in infants with clinical sepsis (N=11) was significantly higher than that of healthy term infants (Figure 3; p=0.014). The plasma BPI concentrations were in cord blood of preterm infants (N=21), 5.5µg/L (1.4-38.6); in cord blood of term infants (N=29), 5.9µg/L (1.2-35.2); in healthy term infants at the age of 2 to 5 days (N=17), 8.9µg/L (3.9-179.0); in healthy adults (N=15), 7.3µg/L (0.7-18.4); and in infants with clinical sepsis (N=11), 27.8µg/L (8.6-883). The difference between these groups was significant (p=0.014, Kruskal-Wallis, ANOVA). There were no blood culture-positive infections among the infants with clinical sepsis.

5.2 PRETERM INFANTS WITH RDS (I)

The mean basal expression of CD11b/CD18 on the surface of neutrophils in cord blood samples of preterm infants (N=11) with RDS was significantly lower (83 RFU at SD 21 *vs*. 112 RFU at SD 38; 95% CI for difference 5 to 53) than that of term infants (N=30; Figure 4). In preterm infants with RDS, CD11b/CD18 expression level on the first postnatal day (N=29; 165 RFU at SD 53) was significantly higher than the respective levels on neutrophils from cord blood of preterm infants (difference 82 RFU, 95% CI 59 to 106) and on neutrophils from 1-day-old preterm infants without RDS (N=12; difference 59 RFU, 95% CI 17 to 90). During follow-up, CD11b expression levels in preterm infants with RDS decreased significantly (p-value for linear trend < 0.001). On postnatal days 2 to 5, neutrophil CD11b/CD18 expression was significantly higher in preterm infants with RDS than in term infants (N=17; p<0.001). Basal CD11b/CD18 expression levels of neutrophils in cord blood samples from term infants, in postnatal samples from term infants at days 2 to 5, and in samples from healthy adults (N=25) were comparable (p=0.95).

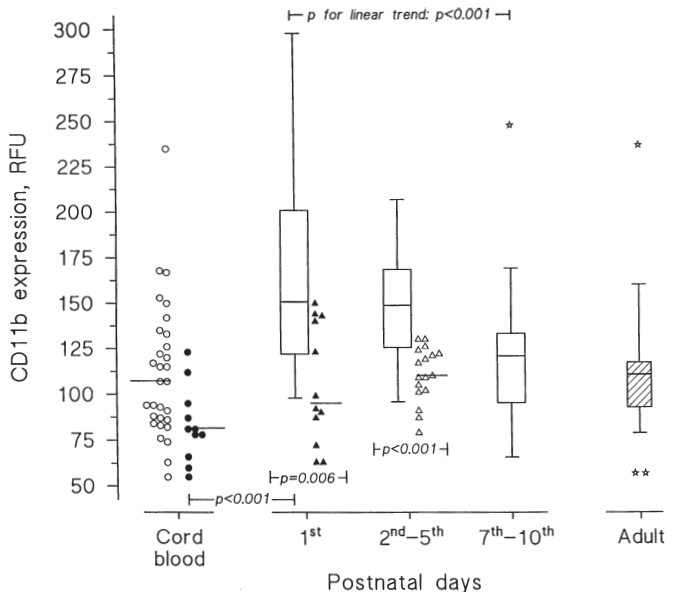


Figure 4. Basal CD11b/CD18 expression on neutrophils in cord blood samples from healthy term infants (open circles, n = 30) and preterm infants (closed circles, n = 11), in postnatal samples from preterm infants with RDS (open box and whiskers; n = 29 at each time point), preterm infants without RDS on day 1 (closed triangles, n=12), and from healthy term infants on days 2 to 5 (open triangles, n = 17), and in adult healthy volunteers (shaded box and whiskers; n=25). All blood samples were cooled on ice immediately after sampling and processed for flow cytometry. The box indicates 75th and 25th percentiles with the central line the median. Whiskers represent the range with outliers indicated by asterisks. From preterm infants with RDS, at least one postnatal sample was obtained at each of the three time points; if additional samples were obtained, arithmetic mean of CD11b/CD18 density was calculated at days 2nd - 5th and 7 th - 10 th, and, finally, the course of CD11b/CD18 expression was evaluated using linear trend test. Neutrophils in aliquots of some postnatal samples, some cord blood samples, and some adult blood samples were stimulated with FMLP.

5.3 DEXAMETHASONE TREATMENT (IV)

5.3.1 Clinical variables

No significant differences existed between the groups in prenatal or postnatal factors before the introduction of dexamethasone therapy (IV: Table 1). Maximal FiO2 on day 1, before entry into the study (70±24 % vs. 74±23 %), as well as the numbers of surfactants were comparable between groups. Duration of mechanical ventilation tended to be shorter in the DEX group than in the CONTROL group (3.6±2.7 vs. 6.8±5.6 days; p=0.17). One infant in the DEX, and seven in the CONTROL group developed BPD, as defined as requiring additional oxygen at 36 weeks´ corrected postnatal gestational age with abnormal radiologic pulmonary findings (Shennan et al., 1988; p=0.01). Intraventricular hemorrhage (IVH) (Grade I-III) was present in five in the DEX, and in three in the CONTROL group. No differences were found in postnatal infections (culture-proven or clinical sepsis) between the groups during the first week of life. In the DEX group, three infants needed insulin for hyperglycemia, and three needed antihypertensive medication. In the CONTROL group, neither hyperglycemia nor hypertension was found. Gastrointestinal bleeding was evident in one infant in the DEX group during the second indomethacin course at the age of 4 days. Two infants died during the neonatal period: one in the DEX group succumbed to severe intraventricular hemorrhage at the age of 4 days, and one in the CONTROL group to intestinal volvulus at the age of 9 days. Follow-up at the age of 3 to 12 months of age showed neurologic abnormalities in 4 infants in the DEX group, and in 5 in the CONTROL group. In the DEX group, 2 had mild hemiplegia, one had spasticity of the lower extremities, and one had increased muscle tone in the shoulder region. In the CONTROL group, 2 infants had spastic diplegia, one had mild hemiplegia, and 2 showed developmental delay (IV: Table 2).

5.3.2 Phagocyte activation

On day 1, adhesion-molecule expression values were comparable between the DEX group (N=15) and CONTROL group (N=15; Figure 5a). On days 2 to 3 in the DEX group, neutrophil CD11b/CD18 expression level, 100 RFU (70-190) *vs.*154 (96-213) and monocyte CD14 expression level, 235 RFU (102-433) *vs*. 355 RFU (219-533) both were lower than in the CONTROL group (both p=0.01) (Figure 5a, and Figure 6c).

On days 2 to 3 in the DEX group, the monocyte L-selectin expression level was lower than in the CONTROL group, 150 RFU (63-259) *vs*. 230 RFU (108-316) (p=0.05) (Figure 6b). During the same time period, the neutrophil L-selectin expression level in the DEX group was 104 RFU (81-209) *vs*. 182 RFU (74-264) in the CONTROL group; p=0.08.

On days 5 to 7 in the DEX group, the monocyte L-selectin expression level was lower than in the CONTROL group, 111 (37-143) *vs*. 185 (42-267); p=0.04 (Figure 6b).

In the DEX group on day 3, P- MIP-1- a was lower than in the CONTROL group, 20 ng/L (20-32) vs. 37 ng/L (20-70); p=0.005. On day 7, P- MIP-1-a levels were comparable. No difference existed during the study period between the groups in monocyte CD11b expression levels (Figure 6a).

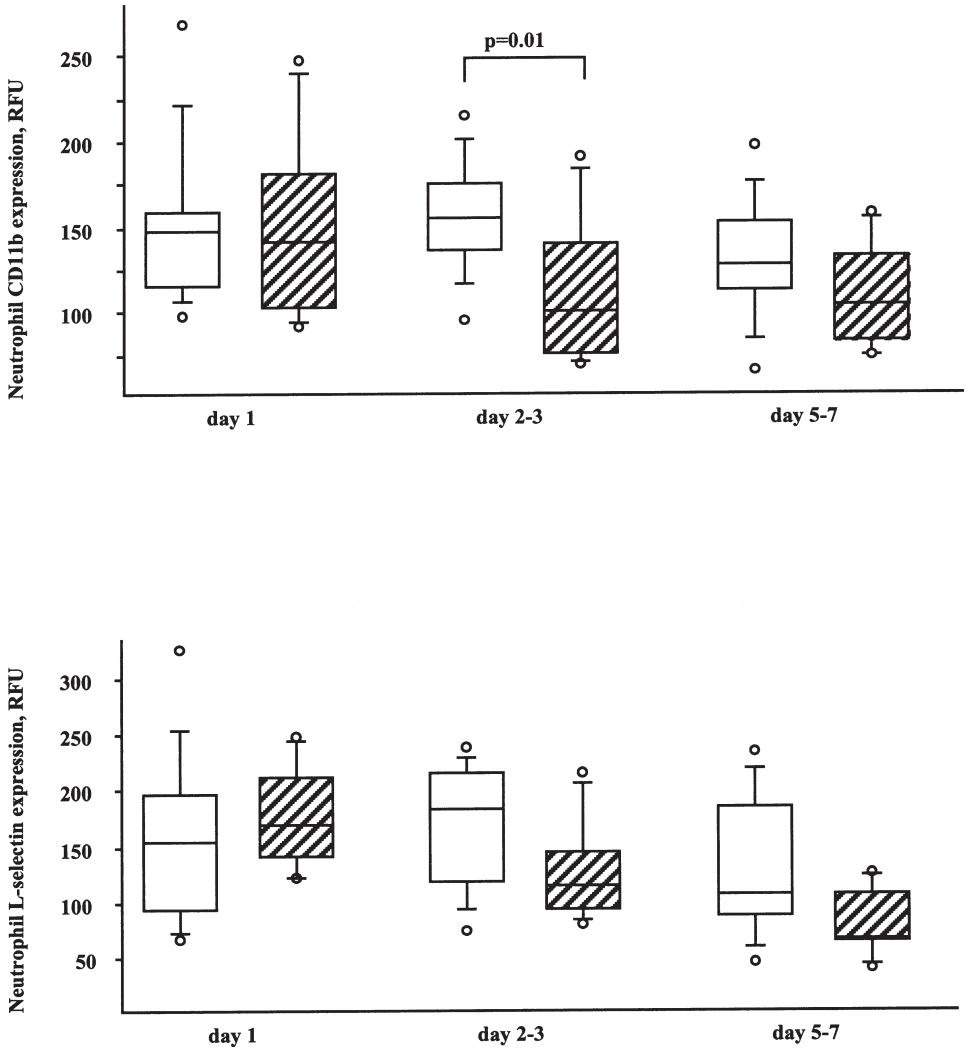
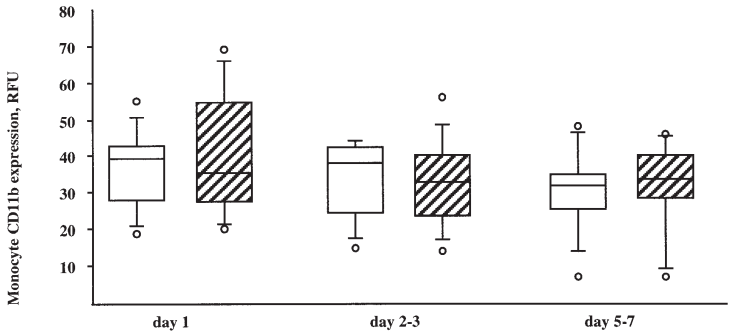
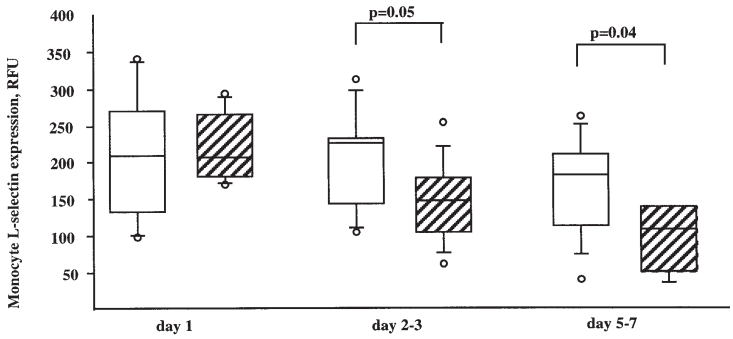


Figure 5. Box and whiskers plots of neutrophil CD11b/CD18 (5 a) and L-selectin (5 b) expression levels on day 1, on days 2-3, and on days 5-7 in preterm infants receving early dexamethasone treatment (hatched boxes), and in controls (white boxes). The box indicates 75th and 25th percentiles with the central line the median. Whiskers represent the range with the outliers indicated by asterisks.

a)



b)



c)

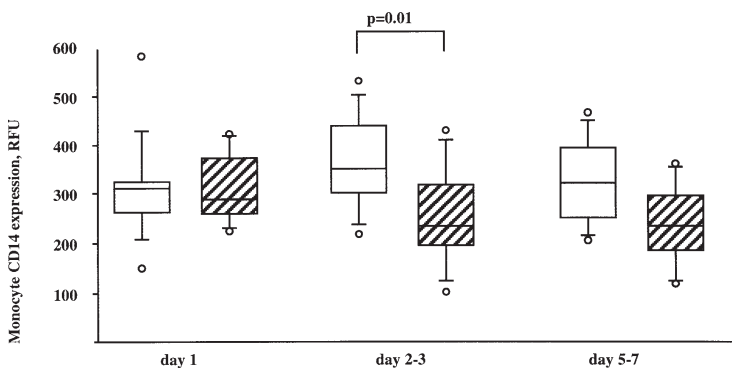


Figure 6. Box and whiskers plots of monocyte CD11b/CD18 (6 a), L-selectin (2 b), and CD14 (6 c) expression levels on day 1, on days 2-3, and on days 5-7 in preterm infants receiving early dexamethasone treatment (hatched boxes), and in controls (white boxes). The box indicates 75th and 25th percentiles with the central line the median. Whiskers represent the range with the outliers indicated by asterisks.

5.4 GRANULE PROTEIN RELEASE (I, III)

5.4.1 FMLP-stimulated neutrophil CD11b/CD18 expression (I, III)

Levels of FMLP-stimulated CD11b/CD18 expression of neutrophils from preterm infants with RDS remained virtually unaltered during the follow-up period (Table 5). The FMLP-stimulated CD11b/CD18 expression level of neutrophils from preterm infants with RDS aged one day was similar to that from term cord blood (post hoc: $p=0.65$), but significantly lower than that from adults (post hoc: $p<0.001$) (ANOVA: $p<0.001$). The mean fold-increase in CD11b/CD18 expression of cord blood neutrophils from term infants (n=28) was higher than that of neutrophils from preterm infants on days 7 to 10 (n=16) (9.4 at SD 3.3 *vs*. 7.0 at SD 1.8) and lower than that of adult neutrophils (n=23) (14.2 at SD 4.7) (ANOVA: $p<0.001$; all post hoc comparisons: $p<0.01$).

FMLP-stimulated PMN CD11b/CD18 expression in preterm cord blood, 1071 RFU (552-1286) and in term cord blood, 918 (567-1472), was on the same level, but significantly lower than that in adult blood, 1592 (973-1946) (Kruskal-Wallis; $p<0.001$).

Table 5. CD11b/CD18 expression on neutrophils after FMLP-stimulation.

	Number	CD11b/CD18 expression, RFUx10^2 Mean (SD)
Term cord blood	28	9.5 (2.0)
Preterm postnatal days	16	
1st		8.8 (2.2)
2nd-5th		7.4 (1.7)
7th-10th		8.4 (1.4)
Adults	23	14.7 (2.6)

Anova between groups, $p<0.001$; term cord vs. adults, $p<0.001$; preterm vs. adults, each $p<0.001$.

5.4.2 PMA-stimulated release of BPI and MPO release (III)

After stimulation with PMA, the BPI concentrations of the culture supernatants were 11.1µg/L (6.5-66.7) in preterm cord samples, 81.0µg/L (35.0-377.0) in term cord samples, and 41.4µg/L (28.6-82.1) in adult samples. The differences between these groups were significant (p=0.001, ANOVA; preterm cord *vs*. term cord, p=0.001; term *vs*. adult, p=0.006). A positive correlation appeared between concentration of BPI and PMN count (r=0.60, p=0.0001).

The BPI/PMN ratio (ng /10^6 PMN) for preterm cord blood was significantly lower than that for healthy adults (8.8 *vs*. 23.4; p=0.024) (Figure 7).

After stimulation with PMA, the concentrations of MPO in the culture supernatans were 28.5 µg/L (12.0-42.0) in preterm cord samples (N=21), 147.5 µg/L (20.0-783.0) in term cord samples (N=29), and 43.0 µg/L (20.0-248.0) in adult samples (N=15**,** p=0.0002, Kruskal-Wallis; preterm cord *vs*. term cord, p=0.0002; term *vs*. adult, p=0.018; preterm *vs*. adult, p=0.013).

The MPO/PMN ratios (ng /10^6 PMN) for preterm 20.0 (11.3-46.7), for term 19.0 (2.2-223.7), and for adult blood samples 27.8 (9.1-80.7) were comparable (p=0.67).

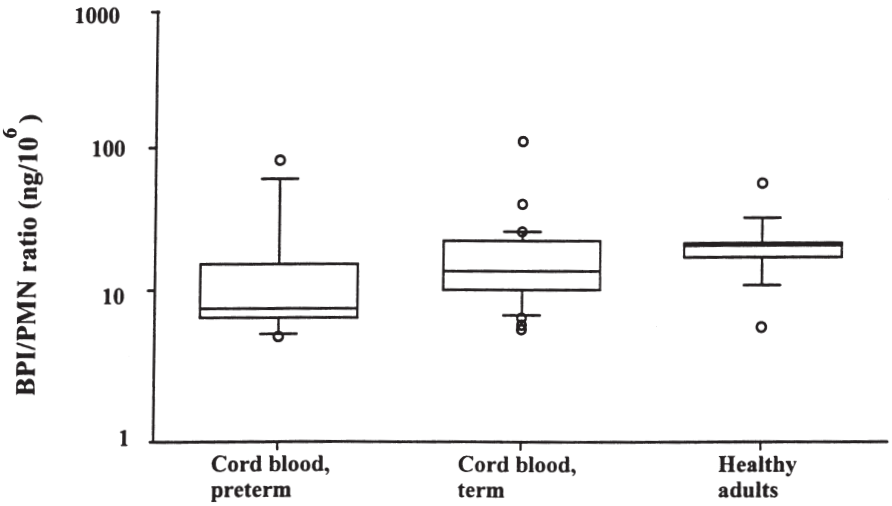


Figure 7. Box and whiskers-plot of extracellular BPI/PMN ratio in culture of whole blood supplemented with PMA. The box indicates 75th and 25th percentiles with the central line the median. Whiskers represent the range with outliers indicated by open circles. Cord samples from preterm infants (n=8) and term infants (n=29), healthy adults (n=15); p=0.043 between the groups; p=0.024, preterm cord *vs.* adults.

6. DISCUSSION

6.1 neutrophil CD11b/CD18 as a marker of systemic inflammation (I, II, III, IV)

Wheter an increased CD11b/CD18 on neutrophils indicates neutrophil activation and systemic inflammation in newborn infants as in adults (Takala *et al*., 1999a, Takala *et al*., 1999b), is an important question. In this project, in newborn infants born at or near term with early-onset sepsis, and in preterm infants with RDS, an increased expression of neutrophil CD11b/CD18 was observed during the early course of the disease. Levels of CD11b/CD18 expression in both groups were comparably high, the highest values, however, being found in infants with positive blood culture results.

6.1.1 Sepsis

Sepsis syndrome, a clinical diagnosis defined as a systemic response to infection (Bone *et al*., 1992) is commonly suspected in neonatal care. Despite the facts that the total cellular content of CD11b/CD18 is lower in newborn infants (Abughali *et al*., 1994; McEvoy *et al*., 1996), and their PMNs show a lower expression of CD11b/CD18 after stimulation with FMLP than adults, (Andersson *et al*., 1987; Bruce et al., 1987; Andersson et al., 1990; Jones *et al*., 1990; Smith *et al*., 1990; Smith JB *et al*., 1991; Carr *et al*., 1992b; Török *et al*., 1993), neutrophils from newborn infants with blood-culture-positive sepsis in this study were able to increase CD11b/CD18 expression two-to fourfold. This increase is similar to that previously observed in newborn infants (Weirich *et al*., 1998) and in adults with sepsis. In addition, production of the cytokines by neonate monocytes is depressed *in vitro* (Schibler *et al*., 1992), indicating functional immaturity of the innate immune system. However, this study and previous studies show high circulating levels of IL-6 and IL-8 in septic newborns (Buck *et al*., 1994; Berner *et al*., 1998; Franz *et al*., 1999; Berner *et al*., 2000). These data support the conclusion that in septic newborn infants, the elevated level of CD11b/CD18 expression and of plasma IL-8 denote systemic inflammation.

Neutrophil CD11b/CD18 expression level correlated positively with IL-8 concentration in infants with sepsis, and the sensitivity and specificity of these markers were considerably higher than those of CRP. In infants born at or near term, determining CD11b/CD18 expression and circulating levels of IL-8 provides a means to distinguish septic infants with systemic inflammation from symptomatic infants who have a non-infective disorder without systemic inflammation. These two assays may therefore serve as useful markers of early-onset neonatal infection in infants born at or near term.

6.1.2 RDS

Signs of early activation of circulating neutrophils appear in preterm infants with RDS who show no evidence of infection (Brus *et al*., 1996; Gaylord *et al*., 1996; Brus *et al*., 1997; Sarafidis *et al*., 2001). In this study, irrespective of risk factors or signs of infection, the blood sample from each preterm infant with RDS had a higher CD11b/CD18 level during the first day of life (I, IV), than the values for the same infant´s cord blood level or than the values for healthy preterm infants. The levels were comparable to the levels of septic, but blood-culture-negative term newborn

infants. This finding suggests that the increased CD11b/CD18 expression on neutrophils probably does not represent a normal physiological adaptation of birth, but rather suggests an early neutrophil activation and systemic inflammation. The increased CD11b/CD18 correlated neither with asphyxia, with morbidities of the mother, nor with the mode of delivery. However, there was a modest correlation of CD11b/CD18 expression with the number of doses of surfactant and maximal FiO_2, a finding that indicates an association between neutrophil activation and the degree of pulmonary morbidity.

Several possible factors may account for neutrophil activation and systemic inflammation in infants with RDS. Barotrauma secondary to positive pressure ventilation, and hyperoxia in alveoli with sufficient ventilation (Rodell *et al*., 1987) can result in damage to the pulmonary endothelium, possibly by activating circulating neutrophils and by increasing migration of these cells into the lungs. Increased neutrophil CD11b/CD18 has, indeed, been shown to be associated with endothelial cell injury and lung damage (Fujita *et al*., 1994). The administration of natural porcine surfactant may play a role in neutrophil activation. Such a role, however, is controversial, because porcine surfactant has both anti-inflammatory and pro-inflammatory properties (Speer *et al*., 1991; Moya *et al*., 1993; Baur *et al*., 1998; Brenner *et al*., 2000). In any case, whether neutrophil activation induces lung injury in RDS, or is the result of it, the correlation of systemic neutrophil activation with pulmonary morbidity observed in this study is in line with previous findings in preterm infants with RDS indicating accumulation of PMNs in the lung tissue during the first postnatal days (Merritt *et al*., 1981a; Merritt *et al*., 1981b; Merritt *et al*., 1983; Ogden *et al*., 1983; Ogden *et al*., 1984; Speer *et al*., 1993).

6.2 Dexamethasone treatment (IV)

The lower expression of CD14 antigen on circulating monocytes in infants who received dexamethasone is in harmony with previous findings (Nockher and Scherberich, 1997). The clinical significance of the depressed CD14 expression level observed in this study is, however, unclear. High CD14 density is related to high monocyte TNF production induced by LPS (Kreutz *et al*., 1999; Louis *et al*., 1998). The lower expression of CD14 antigen possibly results in a depressed monocyte responsiveness to LPS in those infants receiving dexamethasone.

In previous reports, the effects of glucocorticoids on the expression of CD11b/CD18, and L-selectin are diverse. Administration of glucocorticoid to preterm infants at risk for BPD downregulates neutrophil L-selectin expression but leaves CD18 expression levels unaffected (Waisman *et al.*, 1998). In addition, in humans *in vivo*, glucocorticoids downregulate L-selectin on resting neutrophils (Jilma *et al.*, 1997), and blunt neutrophil CD11b/CD18 upregulation in patients undergoing cardiopulmonary bypass (Hill *et al.*, 1994). *In vitro*, dexamethasone downregulates L-selectin and lowers CD11b/CD18 expression levels on activated neutrophils only (Filep *et al*., 1997). In animal experiments, dexamethasone reduces L-selectin expression on circulating neutrophils, and either reduces or has no effect on CD11b/CD18 expression (O´Leary *et al*., 1996; Burton *et al*., 1995). Study IV showed a tendency toward a lower L-selectin expression level on phagocytes in infants treated with dexamethasone. However, to show significant downregulation of L-selectin, the number of newborn infants may have been too small. We interrupted to randomize infants to the study earlier than we originally intended, because during the study period, dexamethasone administration to preterm infants was reported to cause serious long-term neurologic side-effects such as CP (Yeh *et al*., 1997; O´Shea *et al*., 1999; Shinwell *et al*., 2000).

Previous data show that pulmonary inflammation responses are decreased after dexamethasone treatment in preterm infants at risk for BPD (Groneck *et al*., 1993). In this study, the incidence of BPD was lower among those infants who received dexamethasone. This effect may be due to their attenuated phagocyte activation and pulmonary inflammation during the early postnatal period. The finding of this lower incidence of BPD may be explained in part by the exclusion of the most immature infants, in whom developmental rather than inflammatory processes may play a more important role in pathogenesis of chronic lung injury, and in whom glucocorticoids can disturb alveolarization of the developing lung tissue (Jobe, 1999; Massaro and Massaro, 1996).

6.3 Release of phagocyte granule contents

The reduced ability of PMNs from preterm and term infants to enhance CD11b/CD18 expression compared with the ability evident in adults, is in harmony with previous findings of lower CD11b/CD18 expression levels in newborn infants after FMLP stimulation (Andersson *et al*., 1987; Bruce *et al*., 1987; Andersson *et al*., 1990; Smith *et al*., 1990; Smith JB *et al*., 1991; Carr *et al*., 1992b; Török *et al*., 1993). This can be explained by the correlation of the content of CD11b/CD18 with gestational age, and by the lower total cell content of CD11b/CD18 in newborn infants than in adults (Abughali *et al*., 1994; McEvoy *et al*., 1996). In addition, the intracellular signaling mechanisms responsible for the exocytosis of CD11b/CD18 from these vesicles may be immature in newborn infants, because secretory vesicles are formed at a late stage of neutrophil maturation (Borregaard and Cowland, 1997).

In septic infants, the concentration of another granule protein, BPI, which is derived from azurophil granules, was elevated; levels were nearly comparable to those in older children with sepsis syndrome (Wong *et al*., 1995) and in adults with bacteremia (Froon *et al*., 1995) or pneumonia despite the fact that the total intracellular BPI content of term newborn PMNs is lower than that of adult PMNs (Levy *et al*., 1999). In the stimulation assay, however, the amount of BPI/PMN released after in vitro-stimulation with PMA was lower in preterm infants than in term infants or in adults. The finding that the ability of newborn PMN to release MPO is comparable to that of adults is in harmony with the findings that microbial killing ability of a newborn‘s PMNs is as effective as in adults (Conly and Speert, 1991). The diminished BPI release is therefore most likely to be specific to this patient population and may reflect diminished intracellular BPI stores rather than a global impairment in release of azurophil granule contents.

Although the clinical importance of these findings requires further investigation, this study raises the possibility that diminished stimulated CD11b/CD18 expression on neutrophils in both preterm and term newborn infants and BPI release in preterm infants contribute, at least in part, to their increased susceptibility to infections.

CONCLUSIONS

This study suggests that in newborn infants, systemic responses can be triggered by inflammatory stimuli of a diverse nature. In newborn infants born at or near term with sepsis or in preterm infants with RDS, the expression of CD11b/CD18 on neutrophils increases in the early phase of the disease, denoting systemic inflammation.

The determination of CD11b/CD18 may provide a means to distinguish septic infants born at or near term from infants who are symptomatic for reasons other than infection and have no systemic inflammation. These two assays may therefore serve as useful markers of early-onset neonatal infection in infants born at or near term.

Repeated measurements of CD11b/CD18 within few hours may also aid in the decision whether or not to treat with antimicrobials.

In preterm infants with RDS, neutrophil activation as a sign of systemic inflammation is present from the first day of life. This activation may enhance pulmonary inflammation and play a part in the pathogenesis of RDS.

In preterm infants with RDS, dexamethasone treatment causes a transient reduction in systemic inflammation, which may be associated with suppressed pulmonary inflammation and reduce the severity of BPD.

Preterm and term infants have a lower capacity to enhance expression of neutrophil CD11b/CD18 upon stimulation than do adults. The ability of neutrophils to release BPI, on the other hand, is lower only in preterm infants´ neutrophils; neutrophils from term infants release as much BPI as do neutrophils from adults. The ability of neutrophils to release MPO is comparable between preterm and term infants and in adults. These findings may at least in part explain the higher susceptibility of newborn infants to infections.

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